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Drought tolerance of transgenic rice overexpressing maize *C₄-PEPC* gene related to increased anthocyanin synthesis regulated by sucrose and calcium

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Abstract

In order to reveal the role of sucrose (Suc) in early drought response in plants, transgenic rice (*Oryza sativa* L.) plants overexpressing the maize (*Zea mays* L.) C₄-phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) gene (*C₄-pepc*) (PC) and their untransformed wild type (WT) were used under 12 % (m/v) polyethylene glycol 6000 to simulate drought conditions. The results showed that PC has higher relative water content, the increased Suc content, and anthocyanin accumulation than WT during PEG treatment. By spraying 1 % Suc and 1 % Suc non-metabolic analog, turanose, on these plants, Suc in PC leaves increased anthocyanin content and Ca²⁺ content. Further experiments using the Ca²⁺ chelator (EGTA), Ca²⁺ channel antagonist (ruthenium red), and abscisic acid inhibitor (nordihydroguaiaretic acid), showed that, in PC plants, Suc content is closely related to the expression of *sucrose nonfermenting-1-related protein kinases 2* (*SnRK2s*) such as *SAPK8*, *SAPK9*, and *SAPK10* via abscisic acid, and the *SnRK3* such as *SnRK3.1*, *SnRK 3.4*, and *SnRK3.21* via Ca²⁺ and *calcineurin B-like* as well. Furthermore, the target genes associated with anthocyanin synthesis *phenylalanine ammonia lyase*, *chalcone isomerase*, *chalcone synthase*, *flavonoid-3-hydrogenase*, *flavonoid-3'-hydrogenase*, *dihydroflavonone reductase*, and *anthocyanin synthase*, their regulated genes *basic helix-loop-helix (bHLH) proteins OsB1* and *OsB2*, *R2R3-MYB transcription factor OsC1*, and some transcription factors (*constitutively photomorphogenic 1*, *elongated hypocotyl 5*, and *purple acid phosphatase gene 2*) in PC plants also increased via Suc and Ca²⁺ during the PEG treatment. Some Suc transporter genes *OsSUT1* and *OsSUT5* in PC lines during PEG treatment further showed an enhancement for the function of the signal of Suc. Thereby, increasing anthocyanin biosynthesis via Suc and Ca²⁺ signaling cascade is one of the important mechanisms on drought tolerance in the PC lines.

Additional key words: anthocyanin; calcium, *C₄-pepc*, drought response, rice, SnRK, sucrose, *Oryza sativa*.

Introduction

The C₄ cycle serves as a 'CO₂ pump' that concentrates

CO₂ in the leaf and suppresses photorespiration (Dai *et al.* 1993). Under conditions of drought, high temperature, and nitrogen or CO₂ limitation, C₄ plants

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Abbreviations: ABA - abscisic acid; ANR - anthocyanin reductase; ANS - anthocyanin synthase; bHLH - basic helix-loop-helix; CBL - calcineurin B-like; CDPKs - Ca²⁺-dependent protein kinases; CHI - chalcone isomerase; CHS - chalcone synthase; COP1 - constitutively photomorphogenic 1; DFR - dihydroflavonone reductase; EGTA - ethylene glycol diethyl ether diamine tetraacetic acid; ELISA - enzyme-linked immunosorbent assay; F3H - flavonoid-3-hydrogenase; F3'H - flavonoid-3'-hydrogenase; FW - fresh weight; Fru - fructose; Glu - glucose; HY5 - elongated hypocotyl 5; LAR - leucoanthocyanidin reductase; MAPKs - mitogen-activated protein kinases; NDGA - nordihydroguaiaretic acid; *PAP2* - *purple acid phosphatase gene 2*; PAL - phenyl-alanine ammonia-lyase; PA - phosphatidic acid; PC - C₄-phosphoenolpyruvate carboxylase gene transgenic rice type; PEPC - phosphoenolpyruvate carboxylase; PEG 600 0- polyethylene glycol 6000; PPK - pyruvate orthophosphate (Pi) dikinase; qPCR - quantitative polymerase chain reaction.; RWC - relative water content; RR - ruthenium red; Suc - sucrose; SnRK - sucrose nonfermenting-1-related protein kinase; SuS - sucrose synthase; SUT - sucrose transporter; SIAA1 - Suc-induced anthocyanin accumulation; Tur - turanose; TW - turgid weight; WDR - WD-repeat proteins; WT - wild type.

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have a competitive advantage over C_3 plants (Zhu *et al.* 2010). Therefore, introducing the C_4 -type photosynthetic cycle to C_3 plants such as rice to build " C_4 Rice" will be an important scientific strategy to address food security in the future (Von Caemmerer *et al.* 2012). One of the successful approaches to increase rice yield in the present climate scenario involves the introduction of C_4 genes (phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31), pyruvate orthophosphate (Pi) dikinase (PPDK, EC 2.7.9.1), NADP-dependent malate dehydrogenase (NADP-MDH, EC 1.1.1.82), and NADP-dependent malic enzyme (NADP-ME, EC 1.1.1.40) into C_3 plants (Ku *et al.* 1999, Bandyopadhyay *et al.* 2007, Taniguchi *et al.* 2008, Doubnerová Hýsková *et al.* 2014, Sen *et al.* 2017). Among them, most of the transgenic rice plants overexpressing C_4 -PEPC also displayed increased photosynthetic efficiency and tolerance to photo-oxidation, reduced photorespiration, increased drought and low nitrogen tolerance, and a significant increase in grain yield (Jiao *et al.* 2002, Fukayama *et al.* 2003, Bandyopadhyay *et al.* 2007, Taniguchi *et al.* 2008, Gu *et al.* 2013, Lian *et al.* 2014, Chen *et al.* 2014, Ding *et al.* 2015, Huo *et al.* 2017, Tang *et al.* 2018). These results suggest that strengthening C_4 -PEPC genes may be one of the important strategies to increase grain yield and stress tolerance in C_3 plants, synergistically. However, the molecular mechanism of the better balance between high grain yield and stress tolerance in the transgenic rice plants overexpressing C_4 -pepc (the following abbreviations as PC) is still to be established.

Phosphoenolpyruvate carboxylase (PEPC) is a tightly controlled enzyme located at the core of plant C-metabolism that catalyses the irreversible β -carboxylation of PEP to form oxaloacetate and Pi (Chollet *et al.* 1996). The critical role of PEPC in assimilating atmospheric CO_2 during C_4 and Crassulacean acid metabolism photosynthesis has been studied extensively (O'leary *et al.* 2011). Most PEPCs in plant showed allosteric inhibition by malate and activation by glucose 6-phosphate and it also can be regulated by irradiance, sugar, and nutrition condition (Lepiniec *et al.* 1994, Nimmo 2003). Our previous research also showed that PEPC in transgenic rice plant was regulated by phosphatidic acid (PA) (Li *et al.* 2011), H_2O_2 (Ren *et al.* 2014), NO (Chen *et al.* 2014), and Ca^{2+} (Qian *et al.* 2015a,b, Liu *et al.* 2017). The mechanism on how does the transgenic rice overexpressing C_4 -PEPC sense the second messengers is not known.

Sugar, which can act as an osmoprotectant and/or antioxidant, can play a protective role in different plant processes, including growth and development, stress response, and yield formation (Proels and Hückelhoven 2014, Kunz *et al.* 2014, Li and Sheen 2016, Wen *et al.* 2017). More recently, sugar can also be involved in the complex signalling networks with other signals such as plant hormones, nitrogen signals, and optical signals during plant growth and development, but the clear evidence is limited (Sami *et al.* 2016, He *et al.* 2016). Sugars can regulate pyruvate phospho dikinase (PPDK) gene, C_4 malic enzyme gene (*ME1*) and C_4 PEP carboxylase (*PEPC1*) gene in maize (Sheen 1999). Sucrose supply also enhanced PEPC phosphorylation in *in*

vitro *Solanum tuberosum* (Sima and Desjardins 2001). Our previous research has shown that there is an abundance of photosynthetic products such as total soluble sugars in transgenic rice plants overexpressing C_4 -pepc when compared with untransformed rice plants (wild type, WT) during the later growth stages of rice (Li and Wang 2013). PEPC effectively alleviated oxidative damage and enhanced drought tolerance in PC *via* increases in Suc decomposition (Zhang *et al.* 2017a). Furthermore, PC plants were more reliant on the hexokinase sugar signaling pathway than on abscisic acid (ABA) pathway under drought conditions (Wu *et al.* 2017). PEPC has been reported to be involved in the synthesis of secondary metabolites, such as anthocyanins (Xie *et al.* 2016). Our previous research showed that the increased shikimic acid content in flag leaves was found especially in PC leaves (Li *et al.* 2010). DCMU experiments showed that inhibition of photosynthetic electron transfer induces anthocyanin synthesis in PC rice in response to drought (He *et al.* 2018). Much research on anthocyanin induced by sugar have been widely reported in many colored plants (Zhang *et al.* 2014). Whether more content of sugar in PC, especially Suc, is also involved in the regulation of anthocyanin synthesis under drought condition has not been studied.

Sucrose nonfermenting-1-related protein kinase (SnRKs) in plants links the sugar signaling and stress, which has three subfamilies as SnRK1, SnRK2, and SnRK3 (Zhang *et al.* 2017b). SnRK1 expression in PC rice lines is also up-regulated under drought stress conditions (Liu *et al.* 2017). SnRK2 is a plant-specific protein kinase family and key signal transducers in the ABA pathway. SnRK2 family has 10 members (SAPK1-10) in *Oryza sativa* (Hrabak *et al.* 2003). Overexpression of *TaW55a*, *TaSnRK2.4*, *TaSnRK2.7*, *TaSnRK2.8*, or maize *ZmSAPK8* enhanced the tolerance of *Arabidopsis* to drought, 2 % PEG, 5 % glycerol (Xu *et al.* 2009). More recently, our research suggested that PC can decrease the expression of *calcineurin B-like (CBL)* and *SnRK3s* by increasing glucose (Glu), participate in the stomatal regulation *via* NO, maintain relative water content (RWC), keep stable photosynthetic capacity, conferring drought tolerance (Zhang *et al.* 2018). These studies suggested that different *SnRKs* family genes might play important roles in PC rice for drought tolerance.

The aim of this study, was to investigate the interaction between Suc and some signal molecules such as ABA and Ca^{2+} on anthocyanin synthesis in PC lines during drought treatment. This study can help further understanding of the mechanisms underlying drought tolerance in crops and offer a theoretical basis on the high yield and stress tolerance observed in PC rice.

Materials and methods

Plants and experimental conditions: We used the transgenic rice (*Oryza sativa* var. *japonica* L.) line in which the maize *C_4*-pepc gene is over-expressed (named PC). The *C_4*-pepc gene is an 8.8-kb fragment containing

all exons, introns, the promoter (from -1212), and the terminator sequence (about 2.5 kb) of the *pepc* gene (Matsuoka and Minami 1989). We used 11th generation plants (Qian *et al.* 2015b) derived from third generation plants (Ku *et al.* 1999). WT rice plants (cv. Kitaake) were used as the control. All plants were grown in nutrient media consisting of 1.425 mM NH_4NO_3 , 0.323 mM NaH_2PO_4 , 0.513 mM K_2SO_4 , 0.998 mM CaCl_2 , 1.643 mM MgSO_4 , 0.009 mM MnCl_2 , 0.075 mM $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$, 0.019 mM H_3BO_3 , 0.155 mM CuSO_4 , 0.036 mM FeCl_3 , 0.070 mM citric acid, and 0.152 mM ZnSO_4 (Yoshida *et al.* 1976). Plants were maintained in a growth room at 28–30 °C and 70 % relative humidity with a 16-h photoperiod (photosynthetic photon flux density of 400 $\mu\text{mol m}^{-2}\text{s}^{-1}$). The nutrient solution was refreshed every 2 d. When four mature leaves were fully expanded, the plants were harvested for subsequent analyses.

Treatments: To simulate drought conditions, plants were transferred to culture solution containing 12 % (m/v) PEG 6000 (Liu *et al.* 2017). Samples were collected at 0, 2, 4, 8, and 12 h, frozen in liquid nitrogen, and stored at -80 °C for further analyses. For the exogenous sugar treatment, the plants were divided into three groups; one group was sprayed with 1 % Suc nutrient solution (Yoshida *et al.* 1976), one group with 1 % turanose nutrient solution (Yoshida *et al.* 1976), and the control group with distilled water in the dark for pretreatment. The pretreated plants were then transferred to the conditions with 30 °C and a photosynthetic photon flux density of 400 $\mu\text{mol m}^{-2}\text{s}^{-1}$ for 10 h. The photosynthetic parameters of the leaves of the treated plants were measured the following day after being kept in the light for 4 h. The plants were then transferred to culture media containing PEG 6000 and incubated at 400 $\mu\text{mol m}^{-2}\text{s}^{-1}$ for 4 h. Photosynthetic parameters were measured after different treatments.

The plants were cut at the base of the stem and placed in beakers wrapped in aluminum foil containing different treatment solutions [distilled water, 10 mM EGTA, 80 μM ruthenium red (RR) (Qian *et al.* 2015a), or 100 μM nordihydroguaiaretic acid (NDGA)] for 1 h. Samples were then treated with 5 % PEG-6000 for 0–4 h at 28 °C. The samples were collected after 4-h treatment. Distilled water was used as a control. After treatment, the second leaf was collected from each detached seedling, immediately frozen in liquid nitrogen, and stored at -80 °C for further analysis.

Measurement of RWC: To measure the RWC, 10 leaf discs (0.5 cm in diameter) per replicate were removed from the central third of the leaves using a circular cutter. The discs were weighed to determine the fresh mass (FM), and then floated on water at 4 °C in the dark. After 18 h, the water saturated mass (WSM) was measured. After treatment, discs were dried at 75 °C and DM was measured. The RWC (%) was calculated as follows: $\text{RWC} = (\text{FM} - \text{DM}) / (\text{WSM} - \text{DM}) \times 100$ (Qian *et al.* 2015a).

Measurement of net photosynthetic rate: Photosynthetic parameters of rice lines were conducted using an open gas exchange system, equipped with blue- and red-

radiation sources to measure photosynthesis under 1000 $\mu\text{mol m}^{-2}\text{s}^{-1}$ and a 500 mmol s^{-1} flow rate (*LI-6400*; *Li-Cor*, Lincoln, NE, USA) (Li *et al.* 2011). The photosynthetic parameters were also analyzed between 9:00 and 11:00 in outdoor conditions (25–30 °C, a 67–79 % relative humidity, a photosynthetic photon flux density of $1000 \pm 10 \mu\text{mol m}^{-2}\text{s}^{-1}$, and a CO_2 concentration of $390.0 \pm 10.5 \mu\text{mol mol}^{-1}$) using the second leaf of each plant. Each measurement was conducted four times in independent trials, using five replicates per treatment.

Measurement of the total soluble sugar content and content of different sugars: Soluble sugar content determination was performed as described by Li and Wang (2013). Sucrose content was determined using the method of Halhoul and Kleinberg (1972) whereas Glu and fructose (Fru) content was determined as described by Yordanov and Georgieva (2004). The absorbance of the reaction mixtures of Suc, Glu, and Fru was measured at 290, 505, and 285 nm, respectively.

Measurement of proline content and anthocyanin content: The proline content was measured at 520 nm using the method of Bates *et al.* (1973). Anthocyanin content was measured at 657 nm as described by Rabino and Mancinelli (1986).

Measurement of NO, calcium ion, H_2O_2 and ABA content: The NO, calcium ion, and H_2O_2 content in leaves was measured as described by Murphy and Noack (1994), Qian *et al.* (2015b), and Patterson *et al.* (1984), respectively. Absciscic acid (ABA) content was determined by competitive enzyme-linked immunosorbent assay (ELISA) using the protocol of Dong *et al.* (2014). Absciscic acid was detected using an anti-ABA antibody and quantified according to the instructions of the ABA ELISA.

Measurement of PEPC activity: Phosphoenolpyruvate carboxylase (EC 4.1.1.31) activity was measured as described by Giglioli-Guivarch *et al.* (1996).

Extraction of total RNA and real-time quantitative PCR (qPCR): Total RNA was extracted using an RNA simple total RNA kit (*Tiagen*, Beijing, China), according to the manufacturer's instructions. Reverse transcription reactions were conducted using a *TaKaRa PrimeScript RT Master Mix* perfect real time kit (*TaKaRa*, Dalian, China). Real time qPCR analysis was conducted using an *ABI 7300* real-time PCR system (*Applied Biosystems*, Foster City, CA, USA) using the method recommended in the *TaKaRa SYBR Premix Ex Taq II* kit. The reaction mixture contained 10 mm³ of *SYBR® Premix Ex Taq II* (*TaKaRa*), 0.4 mm³ of ROX reference dye (*TaKaRa*), upstream and downstream primers (each 0.1 pmol dm⁻³), 2 mm³ of cDNA template, and ddH₂O to a volume of 20 mm³. The reaction program was as follows: 94 °C for 1 min followed by 40 cycles of 94 °C for 15 s, 60 °C for 45 s, and 72 °C for 30 s. We used the *StepOnePlus* real-time PCR system (v. 2.0, *Applied Biosystems*) for these analyses. The following gene primers

were used for qPCR analysis (Table 4 Suppl.).

Data processing and analysis: Data were analyzed by one-way ANOVA and least significant difference multiple comparison tests ($P < 0.05$) using SPSS 19.0 for Windows statistical software (SPSS., Chicago, IL, USA). The qPCR data were analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Results

In this study, PEPC activity and the respective gene expression in PC rice was significantly higher than in WT under 12 % PEG 6000 treatment while those in WT plants did not change significantly during the similar treatment (Fig. 1A). The relative water content (RWC) of PC and WT plants decreased significantly as treatment time increased, with the RWC of PC lines found to be significantly higher than that of WT after 2 h and 4 h treatment (Fig. 1B). The soluble sugar content increased in both the PC and WT lines with increased time of PEG treatment; the soluble sugar content of PC plants was always significantly higher than that of WT plants (Fig. 1C). Proline content showed a similar trend to that observed for soluble sugar content (Fig. 1D). Similarly, the Suc content showed an upward trend as the plants with increasing time of PEG treatment, with a significantly higher Suc content found in the PC lines when compared with the WT plants (Fig. 1E). There were concomitant changes in Suc and anthocyanin under the PEG treatment and the content of anthocyanin in the PC lines were significantly higher than in WT lines at any PEG treatment time point (Fig. 1F). Glu and Fru showed similar trends, with both decreasing in PEG-treated PC and WT lines when compared with untreated controls. As treatment time increased, there was a slight decrease in the content of both Glu and Fru, with the content in the PC lines always significantly higher than in WT lines (Fig. 1G, H).

Elongated hypocotyl 5 (HY5) is a photomorphogenesis promoting a basic leucine zipper transcription factor that is degraded by constitutively photomorphogenic 1 (COP1) ubiquitin ligase in the darkness. HY5 regulates anthocyanin biosynthesis by inducing the transcriptional activation of the MYB75/PAP1 transcription factor in *Arabidopsis* (Zhang *et al.* 2006, Shin 2013a). In this study (Table 1), the transcription of *OsCOP1* peaked after 30 min of PEG treatment in PC lines and was significantly higher in these lines than in WT plants; however, as the treatment time increased, the difference between WT and PC reduced. The *OsHY5* showed a similar expression pattern to *OsCOP1* in PC lines. At the same time, the regulatory genes *OsB1* and *OsB2* encoded basic helix-loop-helix (bHLH) proteins and *OsC1* encodes an R2R3-MYB transcription factor involved in anthocyanin biosynthesis (Sakamoto *et al.* 2001). *OsB1*, *OsB2*, and *OsC1* had the expression patterns similar to *OsCOP1* and *OsHY5* in PC lines during PEG treatment; their levels in WT plants were also similar during the treatment. The structural genes involved in anthocyanin biosynthesis including

phenyl-alanine ammonia-lyase gene (*PAL*), chalcone isomerase (*CHI*), chalcone synthase (*CHS*), flavonoid-3-hydrogenase (*F3H*), flavonoid-3'-hydrogenase (*F3'H*), dihydroflavonone reductase (*DFR*), anthocyanin reductase (*ANR*), anthocyanin reductase (*LAR*), and anthocyanin synthase (*ANS*) were also measured synchronously. The transcriptions of *OsPAL*, *OsCHI*, *OsCHS*, *OsF3H*, and *OsDFR* showed similar expression patterns to those of the regulatory genes *OsB1* and *OsB2* in PC lines. Those of *OsANS* increased as the treatment time increased in both PC and WT lines but were significantly higher in the PC lines. Conversely, *OsF3'H* expression first increased then decreased in both lines as the treatment time increased, and the expression in WT lines was significantly higher than in the PC lines.

No significant differences in the ABA content in WT and PC lines were observed during the first 2 h of PEG treatment alone (Fig. 2A). However, the PC lines had higher ABA content after 4 - 8 h (Fig. 2A). An ABA synthesis inhibitor, nordihydroguaiaretic acid (NDGA), was used in this study combined with PEG-treatment for 4 h (NDGA+PEG). We observed the decrease in the net photosynthetic rate in both rice lines, but the decrease in PC line was significantly lesser than that in the WT (Fig. 2B) and as well as stomatal conductance (Fig. 2C) after 4 h-PEG treatment alone. NDGA+PEG treatment further decreased both net photosynthetic rate and stomatal conductance in two lines and finally eliminated completely the differences between these lines. The transcriptions of *SAPK8*, *SAPK9*, and *SAPK10* were up-regulated after 2-h-PEG treatment alone only in the PC rice lines (Fig. 2D-F). The NDGA+PEG treatment decreased transcriptions of these genes in PC lines during the PEG treatment and also eliminated completely the differences between two lines during PEG treatment alone. This indicated that ABA is involved in the regulation of *SAPK8*, *SAPK9*, and *SAPK10* in PC lines at the transcription level during the early drought response. Furthermore, we also observed that the transcriptions of *OsB1*, *OsB2*, and *OsC1* showed similar patterns to those of *SAPK8*, *SAPK9*, and *SAPK10* after 4-h PEG treatment alone in PC lines. However, NDGA+PEG treatment only partly decreased the *OsB1*, *OsB2* and *OsC1* expressions in the WT and PC lines and did not eliminate the differences between two lines with higher levels observed in PC rice plants (Fig. 3A-C). This suggests that, in addition to ABA-dependent SnRK2, there might be other factors which can regulate the synthesis of anthocyanin in PC lines under drought conditions.

We further sprayed these plants with 1 % Suc or 1 % turanose (Tur; a Suc non-metabolic analog) to observe the regulation of PEPC activity in this study. Table 1 Suppl. shows that PEPC activity in PC lines was much higher than in WT lines under the PEG treatment alone; PEPC activity was not lower in PC lines after Suc or Tur treatments. However, we did not observe any differences in PEPC activity between the Suc- and Tur-treated PC lines (Table 1 Suppl.). Notably, we observed that Suc mitigated the degree of water loss associated with simulated drought conditions, only in PC lines. Spraying with Suc and Tur decreased the soluble sugar content in both PC and WT

Fig. 1. Changes in phosphoenolpyruvate carboxylase (PEPC) activity (A), relative water content (B), soluble sugar content (C), soluble protein content (D), sucrose (Suc) content (E), anthocyanin content (F), glucose (Glu) content (G) and fructose (Fru) content (H) in the leaves of *C₄*-phosphoenolpyruvate carboxylase gene transgenic rice type (PC) and wild type (WT) plants under PEG 6000-simulated drought treatment. CK - control with distilled water; PEG - 4h-PEG 6000 treatment alone. Data are means $\pm$ SEs from measurements of at least three independent experiments with different letters used to indicate significant differences at $P < 0.05$.

Table 1. Transcripts of *OsCOP1* and *OsHY5*, some key regulative genes and structural gene associated with the anthocyanin biosynthesis pathway in leaves of C₄-phosphoenolpyruvate carboxylase gene transgenic rice type (PC) and wild type (WT) plants under PEG 6000 simulated drought treatment for different times. Data are means $\pm$ SEs from measurements of at least three independent experiments with different letters indicating significant differences at $P < 0.05$. *HY5* is a gene for photomorphogenesis promoting basic leucine zipper transcription factor that is degraded by COP1 ubiquitin ligase in darkness. *OsB1* and *OsB2* are regulatory genes encoding basic helix-loop-helix (bHLH) proteins. *OsC1* is a gene encoding an R2R3-MYB transcription factor involved in anthocyanin biosynthesis. *Phenyl-alanine ammonia-lyase* (*PAL*), *chalcone isomerase* (*CHI*), *chalcone synthase* (*CHS*) *flavonoid-3-hydrogenase* (*F3H*), *flavonoid-3'-hydrogenase* (*F3'H*), *dihydroflavanone reductase* (*DFR*), *anthocyanin reductase* (*ANR*), *leucoanthocyanidin reductase* (*LAR*), and anthocyanin synthase (*ANS*) are structural genes involved in anthocyanin biosynthesis.

Genes	Rice lines	0 min	30 min	60 min	120 min
<i>OsCOP1</i>	WT	1.01 $\pm$ 0.04c	1.47 $\pm$ 0.05a	1.22 $\pm$ 0.04b	1.04 $\pm$ 0.03c
	PC	1.02 $\pm$ 0.03b	1.86 $\pm$ 0.07a	1.02 $\pm$ 0.03b	0.97 $\pm$ 0.02b
<i>OsHY5</i>	WT	1.03 $\pm$ 0.03b	1.01 $\pm$ 0.02b	1.01 $\pm$ 0.02b	1.16 $\pm$ 0.04a
	PC	1.04 $\pm$ 0.03c	2.23 $\pm$ 0.08a	1.64 $\pm$ 0.06b	1.58 $\pm$ 0.05b
<i>OsB1</i>	WT	1.01 $\pm$ 0.02d	1.29 $\pm$ 0.03c	2.63 $\pm$ 0.07a	1.53 $\pm$ 0.04b
	PC	1.02 $\pm$ 0.03d	8.82 $\pm$ 0.09b	9.33 $\pm$ 0.11a	2.55 $\pm$ 0.08c
<i>OsB2</i>	WT	1.01 $\pm$ 0.03d	1.27 $\pm$ 0.04c	2.22 $\pm$ 0.05a	1.98 $\pm$ 0.06b
	PC	1.02 $\pm$ 0.02d	2.53 $\pm$ 0.06b	3.07 $\pm$ 0.07a	2.34 $\pm$ 0.05c
<i>OsC1</i>	WT	1.04 $\pm$ 0.03d	4.31 $\pm$ 0.09b	7.24 $\pm$ 0.08a	2.35 $\pm$ 0.04c
	PC	1.01 $\pm$ 0.02d	8.34 $\pm$ 0.09b	16.50 $\pm$ 0.21a	1.81 $\pm$ 0.06c
<i>OsPAL</i>	WT	1.02 $\pm$ 0.03c	2.52 $\pm$ 0.05a	1.11 $\pm$ 0.04b	1.08 $\pm$ 0.03b
	PC	1.02 $\pm$ 0.04d	4.36 $\pm$ 0.07a	1.16 $\pm$ 0.03b	1.09 $\pm$ 0.04c
<i>OsCHI</i>	WT	1.03 $\pm$ 0.03a	1.04 $\pm$ 0.02a	1.02 $\pm$ 0.03a	1.03 $\pm$ 0.04a
	PC	1.04 $\pm$ 0.03d	2.34 $\pm$ 0.06a	1.35 $\pm$ 0.04b	1.25 $\pm$ 0.03c
<i>OsCHS</i>	WT	1.01 $\pm$ 0.02b	1.04 $\pm$ 0.03b	1.38 $\pm$ 0.05a	1.02 $\pm$ 0.03b
	PC	1.03 $\pm$ 0.02c	3.44 $\pm$ 0.09b	5.74 $\pm$ 0.08a	1.02 $\pm$ 0.03c
<i>OsF3H</i>	WT	1.02 $\pm$ 0.03d	6.36 $\pm$ 0.04a	1.98 $\pm$ 0.04b	1.21 $\pm$ 0.03c
	PC	1.03 $\pm$ 0.02d	9.36 $\pm$ 0.08a	1.45 $\pm$ 0.05b	1.17 $\pm$ 0.04c
<i>OsF3'H</i>	WT	1.01 $\pm$ 0.03d	8.77 $\pm$ 0.08b	12.95 $\pm$ 0.12a	4.35 $\pm$ 0.09c
	PC	1.02 $\pm$ 0.04d	5.59 $\pm$ 0.06b	7.34 $\pm$ 0.11a	2.56 $\pm$ 0.06c
<i>OsDFR</i>	WT	1.03 $\pm$ 0.02b	1.62 $\pm$ 0.04a	1.65 $\pm$ 0.05a	1.05 $\pm$ 0.04b
	PC	1.02 $\pm$ 0.03c	1.89 $\pm$ 0.06b	2.03 $\pm$ 0.05a	1.05 $\pm$ 0.04c
<i>OsANS</i>	WT	1.04 $\pm$ 0.04d	2.47 $\pm$ 0.05c	4.23 $\pm$ 0.06b	7.17 $\pm$ 0.07a
	PC	1.03 $\pm$ 0.02a	3.56 $\pm$ 0.06b	6.79 $\pm$ 0.07b	7.48 $\pm$ 0.05a

lines and Suc treatment resulted in an increase in both of the endogenous Suc content and anthocyanin content in the PC, but not in WT. Glu and Fru content in PC lines was also not affected by these treatments. Neither Suc nor Tur spraying treatment altered ATP content in two rice lines indicated that these applied sugars were not used as substrates for energy production.

In this study, Ca²⁺ content increased significantly in PC rice line after PEG treatment, and further increased when combined with the Suc treatment; this special increase of Ca²⁺ content in PC was not observed after Tur treatment, indicating that Ca²⁺ may also be involved specifically in Suc-mediated regulation of the early drought response in PC lines (Fig. 4A). Here, we found that the PEG treatment alone decreased the NO content in both lines, but NO content restored to pre-PEG treatment after Suc and Tur treatments. No differences of NO content in the same rice lines under the two sugar treatments were found, but NO content (Fig. 4B) as well as H₂O₂ content (Fig. 4C) were still higher in PC lines than in WT under these treatments.

To further verify the role of Ca²⁺ in the synthesis of anthocyanin in PC rice lines, the Ca²⁺ chelator (EGTA) and Ca²⁺ channel antagonist (ruthenium red, RR) were used before the PEG treatment. Three SnRK3s genes, *OsSnRK3.1*, *OsSnRK3.4*, and *OsSnRK3.21* increased significantly during PEG treatment alone, while EGTA and RR treatment eliminated the up-regulation of *OsSnRK3.1*, *OsSnRK3.4* and *OsSnRK3.21* expression in PC lines; no significant differences in WT lines with any of the three treatments were observed (Table 2 Suppl.). These results confirmed that calcium signal positive regulated certain SnRK3 genes such as *OsSnRK3.1*, *OsSnRK3.4*, and *OsSnRK3.21* in PC lines. Calcineurin B-like can sense both Ca²⁺ and sugar signals in plants. But the CBL family itself does not have kinase activity and they must recruit the related protein kinases to form complexes for transmitting signals. Fortunately, SnRK3 has CBL-related protein kinase function and jointly respond to stress by sensing intracellular Ca²⁺ signal and sugar signal (Zhang *et al.* 2017b). The expressions of CBL genes (*OsCBL1*,

Fig. 2. Changes in abscisic acid (ABA) content (A), net photosynthetic rate (B), stomatal conductance (C), and relative expressions of *SAPK8*, *SAPK9*, and *SAPK10* (D-F) in leaves of *C₄*-phosphoenolpyruvate carboxylase gene transgenic rice type (PC) and wild type (WT) plants with a 100 μM ABA inhibitor nordihydroguaiaretic acid (NDGA) after 4h of PEG 6000 simulated drought treatment. CK - control with distilled water; PEG - 4h-PEG 6000 treatment alone; NDGA+PEG - 100 μM NDGA treatment combined with 4h-PEG 6000. Data are means ± SEs from measurements of at least three independent experiments with different letters indicating significant differences at *P* < 0.05.

OsCBL3, and *OsCBL10*) in PC lines were similar as expression of *SnRK3s* under the same treatments (Table 2 Suppl.), indicated the *SnRK3*-*CBL*-related protein kinases *via* Ca^{2+} were also involved in the drought response of PC lines. Obviously, these results are the opposite of the results of *Glu* observed by Zhang *et al.* (2018), where the interaction on *SnRK3* *via* *Suc* and Ca^{2+} has a positive effect in PC lines under drought conditions.

Moreover, the expression of anthocyanin biosynthesis-related *OsB1*, *OsB2*, *OsC1* and *OsPAP2* (*purple acid phosphatase gene 2*) (Table 3 Suppl.) also increased in PEG-treated PC lines similarly as the *CBL* and *SnRK3* genes. At the same time, *OsCHS*, *OsDFR*, and *OsANS* were also regulated by Ca^{2+} . Treatments with EGTA+PEG or RR+PEG significantly reduced their transcriptions. Taken together, these results suggest that Ca^{2+} signaling in PC lines can participate as a part of the positive effect

on regulation of the regulatory and structural genes of anthocyanin synthesis through *CBL*/*SnRK3* during the early drought response.

Under drought conditions, the unique accumulation of sucrose in PC rice lines was synchronized with anthocyanin content, the transcriptions respective genes related to anthocyanin synthesis, suggesting the role of *Suc* as a signal molecule in drought response of PC lines. Therefore, we further followed transcriptions of some gene related to sucrose signaling in plants pretreated with Ca^{2+} inhibitors. The soluble sugar content (Fig.5A), *Suc* content (Fig.5B), the transcription of *OsSUT1* (Fig.5C) and *OsSUT5* (Fig.5D) in PC lines were higher than those in WT, especially under PEG treatment. Pretreated with Ca^{2+} inhibitors, level of the four parameters mentioned above was reduced during PEG treatment. The results indicated that calcium ions, as the upstream component, participates

Fig. 3. Transcript amounts of *basic helix-loop-helix (bHLH)* proteins *OsB1* (A) and *OsB2* (B), and *R2R3-MYB* transcription factor *OsC1* (C) in leaves of *C₄*-phosphoenolpyruvate carboxylase gene transgenic rice type (PC) and wild type (WT) plants with 100 μ M nordihydroguaiaretic acid (NDGA) under PEG 6000 simulated drought treatment. Data are means $\pm$ SEs from measurements of at least three independent experiments with different letters indicating significant differences at $P < 0.05$.

in the gene expression and content of soluble sugars and Suc related to the Suc signaling in PC. We still observed that under inhibitor pretreatments, the levels in PC were still higher than those in WT, indicating that there might be other factors involved in regulation on the Suc signalling.

Discussion

Phosphoenolpyruvate carboxylase in *C₄* plants has been

widely concerned by both scientists and breeders as a key functional gene for improved photosynthetic efficiency and tolerance to drought and low N of *C₃* plants such as rice and wheat (Long *et al.* 2015). Drought stress induces a variety of signal transduction pathways, such as Ca^{2+} , H_2O_2 , NO, and ATP in PC lines, leading to a significant increase in PEPC activity and gene expressions (Bandyopadhyay *et al.* 2007, Ding *et al.* 2015, Liu *et al.* 2015, Qian *et al.* 2015a, Qian *et al.* 2015b, Huo *et al.* 2017, Liu *et al.* 2017). In this paper, Suc in PC lines was involved in the regulation of anthocyanin synthesis via SnRK2s-ABA and SnRK3s- Ca^{2+} -CBL, conferring a stronger drought tolerance.

In plants, sugar functions not only as a substrate for energy production, but also together with hormones, to form an integral part of the plant signaling network regulating stress and defense responses (Li and Sheen 2016). Although sugar signaling has been extensively studied in plant cells, most results obtained from various experimental methods and systems proved that Glu and Suc are the key components of response to different stresses (Dalchau and Kay 2011, Tognetti *et al.* 2013, Van den Ende *et al.* 2014, Daloso *et al.* 2016, Wang *et al.* 2016, Wang *et al.* 2017). However, not all plants exhibit the same responses to different stresses, and there are significant variations between genotypes (Welfare *et al.* 2002). Sucrose is the most common form in which carbon is transported from source tissues via the phloem. When Suc arrives in sink tissues, it is unloaded from the phloem and cleaved by acid invertases both in the cell wall and vacuoles or by Suc synthase (SuS) and neutral/alkaline invertases in the cytosol, chloroplasts, and mitochondria (Barratt *et al.* 2009). There are two sites of the phosphorylation in Suc synthase (S15 and S170, or their equivalent) and the site S15 can be linked to sugar availability through Ca^{2+} -dependent protein kinases (CDPKs) in phosphorylation of Suc synthase (Hardin *et al.* 2004), and/or a sugar-responsive Ca^{2+} -independent SnRKs (Zhang *et al.* 2017a). Previous research indeed observed synchronization with a higher sugar decomposition rate exhibiting higher activities of Suc phosphate synthase, SuS, acid invertase, neutral invertase, and transcript levels of *VIN1*, *CIN1*, *NIN1*, *SUT2*, *SUT4*, and *SUT5* in PC lines during PEG treatment (Zhang *et al.* 2017b). But, these results of PC lines were obtained from leaf disc *in vitro*. In this paper, using the intact rice seedlings, the results show that the high Suc content in PC lines was concomitant with high transcript levels of the SnRK2s and SnRK3s family genes, as a positive effector, during PEG treatment.

Many stress-inducible protein kinase families are activated by ABA and other stress signals. These include mitogen-activated protein kinases (MAPKs) (MAPK Group 2002, Danquah *et al.* 2014), CDPKs (Ludwig *et al.* 2004), and SnRKs (Dong *et al.* 2012). The SnRKs are the key compositions between sugar signaling and stress signaling with members divided into three subfamilies: SnRK1, SnRK2, and SnRK3 (Hanson and Smeekens 2009, Zhang *et al.* 2017a). The SnRK1 is a central integrator of stress and energy signaling in plants causing extensive reprogramming gene transcription and controlling plant growth (Baena-González *et al.* 2007). Sucrose activates

Fig. 4. Changes in Ca^{2+} (A), NO (B), and H_2O_2 (C) content in leaves of C_4 -phosphoenolpyruvate carboxylase gene transgenic rice type (PC) and wild type (WT) plants under sucrose (Suc) and PEG 6000 simulated drought treatment after spraying with 1 % (m/v) exogenous Suc and 1 % (m/v) Suc non-metabolic analog turanose (Tur). CK - control with distilled water; PEG - 4h-PEG 6000 treatment alone; PEG+Suc - pre-spraying with 1 % (m/v) exogenous Suc and then PEG 6000 treatment; PEG+Tur - pre-spraying with 1 % (m/v) exogenous Tur and then PEG 6000 treatment. Data are means $\pm$ SEs from measurements of at least three independent experiments with different letters indicating significant differences at $P < 0.05$.

SnRK1, and this kinase is required for the Suc signal transduction leading to starch synthesis and SuS induction in potato (Tiessen *et al.* 2003). However, KIN10/KIN11 (the members of the SnRK1 subfamily that are the closest relatives of SNF1 and AMPK of yeast and mammals, respectively) activities are repressed by Suc in maize protoplasts. The SnRK1 may be different in autotrophic and heterotrophic tissues and depends on the physiological status of the cells (Baena-González *et al.* 2007). The amountss of SnRK1s in PC lines were higher than in WT in response to drought stress (Liu *et al.* 2017). The

SnRK2s are positive regulators of ABA signaling (Boneh *et al.* 2012). Nitric oxide suppresses ABA signaling in guard cells by S-nitrosylation of SnRK2.6 (also known as OPEN STOMATA1) (Wang *et al.* 2015). But, in this study, we did not observe the different changes of NO and H_2O_2 , but Ca^{2+} between PC and WT during the PEG treatment, particularly in exogenous Suc treatment. Calcium, protein kinases (PKs) (Ohto *et al.* 1995, Iwata *et al.* 1998, Wang *et al.* 2001, Raíces *et al.* 2003) and protein phosphatases (PPs) (Takeda *et al.* 1994, Ohto *et al.* 1995, , Wang *et al.* 2001, Ciereszko *et al.* 2002, Siedlecka *et al.* 2003) are

Fig. 5. Changes in soluble sugar content (A), sucrose content (B) and expressions (C,D) of *OsSUT1* and *OsSUT5* in leaves of 4h-PEG 6000 treatment alone (PC) and wild type (WT) plants with Ca^{2+} and Ca^{2+} release antagonist pretreatments under PEG 6000 simulated drought treatment [Ca^{2+} chelator - 10 mM EGTA; Ca^{2+} channel antagonist - 80 μM ruthenium red (RR)]. CK - control with distilled water; PEG - 4h-PEG 6000 treatment alone; EGTA+PEG - 10 mM EGTA treatment combined with PEG treatment; RR+PEG - 80 μM RR treatment combined with PEG treatment. Data are means $\pm$ SEs from measurements of at least three independent experiments with different letters indicating significant differences at $P < 0.05$.

involved in intracellular Suc signaling processes. The PC can regulate related genes through downstream-dependent protein kinases including *CPK4*, *CPK9*, *SnRK1A*, *OsK24*, and *OsK35* and some transcription factor genes *NAC6* and *bZIP60* via Ca^{2+} signaling during the early drought response (Liu *et al.* 2017). The CBLs in plants can sense Ca^{2+} signaling (Zhu *et al.* 2013) but then must transmit signals only by recruiting SnRK3 (Batistic and Kudla 2009). Calcium B-type phosphatase 1 acts as a calcium signaling-related protein that senses intracellular Ca^{2+} and Glu signaling and regulates stomata by interacting with SnRK3 in response to stress (Sanyal *et al.* 2015, Sibaji *et al.* 2017). Pharmacological tests of sugar and calcium inhibitors in this study also show that Suc had also some relationships with calcium signal on the regulation of anthocyanin synthesis involved in drought tolerance in a CBL-SnRK3-dependent manner.

Anthocyanin accumulation as one of the important stress responses in plant is also concerned for studying the signaling network including sugar and hormones (Teng *et al.* 2005, Das *et al.* 2011, Zhang *et al.* 2014). Members of three protein families, R2R3-MYB

transcription factors, bHLH transcription factors, and WD Repeat Proteins (WDR) interact to form a complex (MBW), which activates anthocyanin biosynthesis (Dong *et al.* 2014, Zhang *et al.* 2014, Shin *et al.* 2016). Sucrose in conjunction with transcription factors, such as MYB75/PAP1, encodes one of quantitative trait loci for Suc-induced anthocyanin accumulation (SIAA1). It is considered to be the most effective inducer of anthocyanin biosynthesis in *Arabidopsis thaliana* (Teng *et al.* 2005, Guo *et al.* 2011). These signals can stimulate kinase cascades by activating SnRK, which can then induce downstream transcription factors such as MYB75 and HY5. The *OsCOP1* and *OsHY5* were selected as positive regulators in rice through the transcriptional activation of MYB75 (Zhang *et al.* 2006, Shin 2013a). In this study, during PEG treatment, the transcripts of *OsCOP1* and *OsHY5* in PC lines were induced more than those in WT and also more than three transcription factor genes (*OsB1*, *OsB2*, and *OsC1*) and six structure genes (*OsPAL*, *OsCHI*, *OsCHS*, *OsF3H*, *OsDFR* and *OsANS*) related to anthocyanin biosynthesis via Suc.

Sucrose can stimulate the production of anthocyanin

Fig. 6. Proposed mechanisms underlying the sucrose (Suc)-mediated regulation of anthocyanin biosynthesis under drought stress. Under drought conditions, of *C*₄-phosphoenolpyruvate carboxylase gene transgenic rice type (PC) lines can accumulate more Suc. Sucrose can then stimulate the generation of NO, Ca²⁺, and abscisic acid (ABA) signals. Calcineurin B-like (CBLs) activate sucrose nonfermenting-1-related protein kinase 3 (SnRK3) by sensing Ca²⁺, and NO activates SnRK2 through ABA. The activated kinases can activate the target genes associated with anthocyanin biosynthesis *phenylalanine ammonia lyase* (*OsPAL*), *chalcone isomerase* (*OsCHI*), *chalcone synthase* (*OsCHS*), *flavonoid-3-hydrogenase* (*OsF3H*), *flavonoid-3'-hydrogenase* (*OsF3'H*), *dihydroflavonone reductase* (*OsDFR*), and *anthocyanin synthase* (*OsANS*) via the transcription factors constitutively photomorphogenic 1 (COP1), myeloblastosis (MYB), elongated hypocotyl 5 (HY5), and basic helix-loop-helix (bHLH) to regulate anthocyanin production. This can help PC plants to alleviate damage by drought stress. Full arrows - activation, dashed arrows - effect not demonstrated.

through SnRK kinase that has a very close relationship with Ca²⁺, NO, and ABA signals (Tossi *et al.* 2011, Van den Ende *et al.* 2014, Sibaji *et al.* 2017). Abscisic acid can regulate the synthesis of anthocyanin through MYBs and other transcription factors (Shimada *et al.* 2006, Van den Ende *et al.* 2014). Previous studies have shown that SnRK2 genes, such as SAPK8, SAPK9, and SAPK10, can respond to osmotic stress in an ABA-dependent manner (Kobayashi *et al.* 2005). In this study, *OsB1*, *OsB2*, and *OsC1* of PC were partly regulated by ABA from the data of ABA inhibitor experiment. Calcium signaling can transmit sugar signaling to anthocyanin biosynthesis by Suc absorption (Shin *et al.* 2013b) that relies on interaction with SnRK3 (Sibaji *et al.* 2017). In this study, Ca²⁺ signaling also increased the transcript amounts of regulatory genes and structural genes related to anthocyanin biosynthesis in PC under drought stress, and the same holds for expressions of Suc transfer genes (*OsSUT1* and *OsSUT5*). These results further show the important role of Ca²⁺ on Suc-induced anthocyanin accumulation response to drought.

It needs to be pointed out that we did not observe any differences in PEPC activity between the Suc- and Tur-treated PC lines indicating that the PEPC activity of the PC lines was regulated by these sugars but not Suc-specific regulation. Coupe *et al.* (2006) found that the nature of the signal transported from CO₂-fed source leaves to the SAM, where stomata development is inhibited, was fully compatible with Suc and proposed that Suc might be involved as a signal molecule in plant responses to elevated CO₂ (Coupe *et al.* 2006). Overexpression of *C*₄-PEPC in rice lines indeed increases CO₂ content in PC

lines (Ku *et al.* 1999), which suggests that the change of CO₂ content, might play roles on the regulation of anthocyanin synthesis via Suc and Ca²⁺. This need to be proved in the future.

In summary, Suc in the PC lines is closely associated with signaling pathways including SnRK2 via ABA, and SnRK3 via Ca²⁺ and CBL. Once drought treatment happened by Suc, SnRK2, and SnRK3 were activated by different signal messages. Some transcription factors (*COP1*, *HY5*, *MYBs*, and *bHLHs*) increased, then these regulatory genes induced higher transcript amounts, therefore an increase in anthocyanin content conferring drought tolerance in the PC lines. Combining the previously published data with the results in this study, we have summarized the drought tolerant mechanism underlying the regulation of anthocyanin biosynthesis via Suc and Ca²⁺ in PC lines (Fig. 6).

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